

## **AMENDMENTS TO THE SPECIFICATION:**

**Please amend the specification as follows:**

**Replace the paragraph beginning at page 4, lines 17-27 with the following amended paragraph:**

In another aspect of the embodiment the target protein can be tumor necrosis factor, tumor necrosis factor receptor, interleukin-1, interleukin-1 receptor, interleukin-2, interleukin-2 receptor, interleukin-4, interleukin-4 receptor, interleukin-5, interleukin-5 receptor, interleukin-12, interleukin-12 receptor, interleukin-13, interleukin-13 receptor, p-selectin, p-selectin glycoprotein ligand, Substance P, the Bradykinins, PSGL, factor IX, immunoglobulin E, immunoglobulin E receptor, CCR5, CXCR4, glycoprotein 120, glycoprotein 41, ~~CD4, hemagglutinin~~ hemagglutinin, respiratory syncytium virus fusion protein, B7, CD28, CD2, CD3, CD4, CD40, vascular endothelial growth factor, VEGF receptor, fibroblast growth factor, endothelial growth factor, EGF receptor, TGF receptor, transforming growth factor, Her2, CCR1, CXCR3, CCR2, Src, Akt, Bcl-2, BCR-Abl, glucagon synthase kinase-3, cyclin dependent kinase-2 (cdk-2), or cyclin dependent kinase-4 (cdk-4).

**Replace the paragraph beginning at page 10, lines 13-17 with the following amended paragraph:**

In another embodiment of the invention, the protease cleaves membrane fusion proteins found on human immunodeficiency virus (HIV), Respiratory Syncytial Virus (RSV), or influenza, inhibiting these virus' ability to infect cells. Without these membrane proteins, these viruses would not be able to infect cells. Therefore, the protease could be used to treat or prevent infection by HIV, ~~RSV~~ RSV or influenza.

**Replace the paragraph beginning at page 25, lines 4-16 with the following amended paragraph:**

In order to change the substrate preference of a given subsite (S1-S4) for a given amino acid, the specificity determinants that line the binding pocket are mutated, either individually or in combination. In one embodiment of the invention, a saturation ~~mutatgenesis~~ mutagenesis technique is used in which the residue(s) lining the pocket is mutated to each of the 20 possible amino acids. This can be accomplished using the Kunkle method (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Media Pa.). Briefly, a mutagenic oligonucleotide primer is synthesized which contains either NNS or NNK-randomization at the desired codon. The primer is annealed to the single stranded DNA template and DNA polymerase is added to synthesize the complementary strain of the

template. After ligation, the double stranded DNA template is transformed into E. coli for amplification. Alternatively, single amino acid changes are made using standard, commercially available site-directed mutagenesis kits such as QuikChange (Stratagene). In another embodiment, any method commonly known in the art for site specific amino acid mutation could be used.

**Replace the paragraph beginning at page 35, lines 4-17 with the following amended paragraph:**

In an embodiment, the protease has an amino acid sequence of one of the scaffolds described herein or one of the mutants of the scaffolds. The protease protein is substantially homologous to one of the scaffolds described herein or one of the mutants of the scaffolds, and retains the functional activity of the protein, yet differs in amino acid sequence due to natural allelic variation or mutagenesis. Accordingly, in another embodiment, the protease comprises an amino acid sequence at least about 45% homologous to the amino acid sequence of one of the scaffolds described herein or one of the mutants of the scaffolds, and retains the functional activity of one of the scaffolds described herein or one of the mutants of the scaffolds. In a preferred embodiment, the protease comprises an amino acid sequence at least about 90% ~~homologous~~ homologous to the amino acid sequence of one of the scaffolds. In another preferred embodiment, the protease comprises an amino acid sequence at least about 95% ~~homologous~~ homologous to the amino acid sequence of one of the scaffolds. In another preferred embodiment, the protease comprises an amino acid sequence at least about 99% ~~homologous~~ homologous to the amino acid sequence of one of the scaffolds.

**Replace the paragraph beginning at page 39, lines 18-24 with the following amended paragraph:**

A protease can be administered to cleave a caspase thereby inhibiting its activity. In a preferred ~~embodiment~~ embodiment, the protease can be administered to cleave caspase-3. The protease can also cleave other proteins involved in apoptosis *e.g.*, human cytochrome c, human Apaf-1, human caspase-9, human caspase-7, human caspase-6, human caspase-2, human BAD, human BID, human BAX, human PARP, or human p53. By cleaving these proteins, the protease thereby inactivates them. In this manner the protease can be used to inhibit apoptosis.

**Replace the paragraphs beginning at page 41, line 12 through page 42, line 3 with the following amended paragraphs:**

The protease rat granzyme B was mutated at Ile 99 to an Alanine using the QuikChange (Stratagene) method of site directed ~~mutatgenesis~~ mutagenesis. DNA primers to

introduce the I99A mutation were: Forward primer: CCA GCG TAT AAT TCT AAG ACA GCC TCC AAT GAC ATC ATG CTG (SEQ ID NO:3) Reverse primer: CAG CAT GAT GTC ATT GGA GGC TGT CTT AGA ATT ATA CGC TGG (SEQ ID NO:5). A polymerase chain reaction was made containing the wild type double stranded DNA, the two primers overlapping the mutation, a reaction buffer, dNTP's and the DNA polymerase. After 30 rounds of annealing and amplification, the reaction was stopped. The enzyme DpnI was added to digest the wild type DNA containing a modified base pair, and the resulting nicked DNA strand is transformed into bacteria. A selection against Zeocin ensures only positive clones with grow. The mutation was confirmed by sequencing the granzyme B gene. The same protocol was used to make the remaining granzyme B mutants, with appropriate changes in the mutagenic primers.

The DNA containing the variant granzyme B proteases was transformed into *Pichia pastoris* X33 cells by the published protocol (Invitrogen) and the positive transformants were selected with Zeocin. The colony was transferred to a 1 L liquid culture and grown to a cell density of greater than OD<sub>600</sub>=1.0. Protein expression was induced by the addition of 0.5% methanol and held constant over 72 hours. To purify the variant protease, the culture was centrifuged and the supernatant collected. Gravity based loading flowed the supernatant over a SP-Sepharose Fast Flow cation exchange column. The column was washed with 50 mM [[Mes]] MES, pH 6.0, 100 mM NaCl, and more stringently with 50 mM MES, pH 6.0, 250 mM NaCl. The protein was eluted with 50 mM MES, pH 6.0, 1 M NaCl and the column washed with 50 mM MES, pH 6.0, 2M NaCl and 0.5 M NaOH. The resulting protease was <90% pure. The final protease was exchanged and concentrated into 50 mM MES, pH 6.0, 100 mM NaCl for storage at 4 °C.

**Replace the paragraphs beginning at page 45, line 4-21 with the following amended paragraphs:**

Freshly isolated neutrophils (PMN) [[were]] are resuspended at  $1 \times 10^7$  cells/ml in RPMI 1640 with 0.2% fetal calf serum (FCS) and incubated with various concentrations of protease, specific for the stalk region of TNF-R1 or TNF-R2. After a 1 to 40 min incubation at 37 °C, protease inhibitors [[were]] are added to stop the reaction and the amount of TNF-R released into the media [[were]] is quantitated using ELISA (Roche).

***TNF cleavage.***

<sup>125</sup>I-TNF (40,000 cpm) ~~[[were]]~~ is incubated with varying concentrations of protease and then samples ~~[[were]]~~ are boiled in SDS-PAGE sample buffer and examined on a 12% polyacrylamide gel. Gels ~~[[were]]~~ are dried and exposed to x-ray film(Kodak) at -70 °C.

***TNF binding assay.***

<sup>125</sup>I-TNF or PMN ~~[[were]]~~ are incubated with varying concentrations of proteases as above. The binding of <sup>125</sup>I-TNF exposed to proteases to normal PMN, or the binding of normal <sup>125</sup>I-TNF to PMN exposed to proteases, ~~[[was]]~~ is quantitated using scintillation. Briefly, 10<sup>5</sup> cells ~~[[were]]~~ are incubated with varying concentrations of <sup>125</sup>I-TNF in 96-well filter plates (Millipore) in the presence of protease inhibitors. Cells ~~[[were]]~~ are then washed three times by vacuum aspiration and then 30μl of scintillation fluid (Wallac) ~~[[was]]~~ is added to each well. Scintillation ~~[[was]]~~ is then counted on a Wallac Microbeta scintillation counter. (Adapted from van Kessel *et al.*, J. Immunol. (1991) 147: 3862-3868 and ~~Porteau~~ Porteu *et al.*, JBC (1991) 266:18846-18853).

**Replace the paragraph beginning at page 46, line 4-15 with the following amended paragraph:**

Fusion of the protease gene to either the gene 3 or gene 8 M13 coat proteins can be constructed using standard cloning methods. (Sidhu, Methods in Enzymology, 2000, V328, p333). A combinatorial library of variants within the gene encoding the protease is then displayed on the surface of M13 as a fusion to the p3 or p8 M13 coat proteins and panned against an immobilized, aldehyde-containing peptide corresponding to the target cleavage of interest. The aldehyde moiety will inhibit the ability of the protease to cleave the scissile bond of the protease, however this moiety does not interfere with protease recognition of the peptide. Variant protease-displayed phage with ~~specifiety~~ specificity for the immobilized target peptide will bind to target peptide coated plates, whereas non-specific phage will be washed away. Through consecutive rounds of panning, proteases with enhanced ~~specifiety~~ specificity towards the target sequence can be isolated. The target sequence can then be synthesized without the aldehyde and isolated phage can be tested for specific ~~hydrolysis~~ hydrolysis of the peptide.

**Replace the paragraphs beginning at page 49, line 7-28 with the following amended paragraphs:**

The cleavage of the tumor necrosis factor receptors 1 and 2 (TNF-R1 and TNF-R2) ~~[[were]]~~ are measured using these techniques. Freshly isolated neutrophils (PMN) ~~[[were]]~~

are resuspended at  $1 \times 10^7$  cells/ml in RPMI 1640 with 0.2% fetal calf serum (FCS) and incubated with various concentrations of protease, specific for the stalk region of TNF-R1 or TNF-R2. After an incubation of 1 to 40 min at 37 °C, protease inhibitors ~~[[were]]~~ are added to stop the reaction and the amount of TNF-R released into the media ~~[[was]]~~ is quantified using ELISA (Roche).

Although the invention has been described with respect to specific methods of making and using enzymes capable of cleaving target polypeptide sequences, it will be apparent that various changes and modifications may be made without departing from the invention.

***Cleavage ~~[[Of]]~~ of TNF.***

$^{125}$ I-TNF (40,000 cpm) ~~[[was]]~~ is incubated with varying concentrations of protease, samples ~~[[were]]~~ are boiled in SDS-PAGE sample buffer and examined on a 12% polyacrylamide gel. The gels ~~[[were]]~~ are dried and exposed to x-ray film(Kodak) at -70 °C.

***TNF Binding Assay. ~~TNF Binding Assay.~~***

$^{125}$ I-TNF or PMN ~~[[were]]~~ are incubated with varying concentrations of proteases as above. The binding of  $^{125}$ I-TNF exposed to proteases to normal PMN, or the binding of normal  $^{125}$ I-TNF to PMN exposed to proteases, ~~was~~ is quantified using scintillation. Briefly,  $10^5$  cells ~~[[were]]~~ are incubated with varying concentrations of  $^{125}$ I-TNF in 96-well filter plates (Millipore) in the presence of protease inhibitors. Cells ~~[[were]]~~ are washed three times by vacuum aspiration and 30  $\mu$ L of scintillation fluid (Wallac) ~~[[was]]~~ is added to each well. Scintillation ~~[[was]]~~ is counted on a Wallac Microbeta scintillation counter. (Adapted from van Kessel *et al.*, J. Immunol. (1991) 147: 3862-3868 and ~~Porteau~~ Porteu *et al.*, JBC (1991) 266:18846-18853).